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Detailed Description

1. Title of the Invention

Novel polypeptide

2. Patent Claims

1. A polypeptide represented by the following amino acid sequence:

(Met)n Thr Pro Leu Gly Pro Ala Ser Ser Leu
Pro Gln Ser Phe Leu Leu Lys Cys Leu Glu Gln
Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu
Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys
His Pro Glu Glu Leu Val Leu Leu Gly His Ser
Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys
Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu
Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln
Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro
Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu
Asp Val Ala Asp Phe Ala Thr Thr Ile Trp Gln
Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu
Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala
Ser Ala Phe Gln Arg Arg Ala Gly Gly Val Leu

Val Ala Ser His Leu Gln Ser Phe Leu Glu Val
Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro

(wherein n is 0 or 1).

2. The polypeptide described under Patent Claim 1, characterized by the fact that it is a polypeptide having the activity of a human granulocyte colony-stimulating factor.

3. A recombinant vector containing a gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor.

4. The recombinant vector described under Patent Claim 3, characterized by the fact that the gene is a DNA complementary to a messenger RNA which is obtained as a 15-17S fraction by sucrose density gradient centrifugation and encodes the polypeptide having the activity of a human granulocyte colony-stimulating factor.

5. The recombinant vector described under Patent Claim 3, in which the gene encoding the polypeptide having activity of a human granulocyte colony-stimulating factor encodes the polypeptide sequence shown below or a part of it:

Met Ala Gly Pro Ala Thr Gln Ser Pro Met Lys
Leu Met Ala Leu Gln Leu Leu Leu Trp His Ser
Ala Leu Trp Thr Val Gln Glu Ala Thr Pro Leu
Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu
Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln
Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys
Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu
Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp
Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu
Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala
Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr
Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe
Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu
Gly Met Ala Pro Ala Leu Gln Pro Thr Gln Gly
Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg
Arg Ala Gly Gly Val Leu Val Ala Ser His Leu
Gln Ser Phe Leu Glu Val Ser Tyr Arg Val Leu
Arg His Leu Ala Gln Pro

6. The recombinant vector described under Patent Claim 3, in which the gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor encodes the polypeptide sequence shown below or a part of it:

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met
Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro
Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
Arg Val Leu Arg His Leu Ala Gln Pro

7. The recombinant vector described under Patent Claim 3, in which the gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor encodes the nucleotide sequence shown below or a part of it:

ATG GCT GGA CCT GCC ACC CAG AGC CCC ATG AAG

CTG ATG GCC CTG CAG CTG CTG CTG TGG CAC AGT

GCA CTC TGG ACA GTG CAG GAA GCC ACC CCC CTG

GGC CCT GCC AGC TCC CTG CCC CAG AGC TTC CTG

CTC AAG TGC TTA GAG CAA GTG AGG AAG ATC CAG

GGC GAT GGC GCA GCG CTC CAG GAG AAG CTG TGT

GCC ACC TAC AAG CTG TGC CAC CCC GAG GAG CTG

GTG CTG CTC GGA CAC TCT CTG GGC ATC CCC TGG

GCT CCC CTG AGC AGC TGC CCC AGC CAG GCC CTG

CAG CTG GCA GGC TGC TTG AGC CAA CTC CAT AGC

GGC CTT TTC CTC TAC CAG GGG CTC CTG CAG GCC

CTG GAA GGG ATC TCC CCC GAG TTG GGT CCC ACC

TTG GAC ACA CTG CAG CTG GAC GTC GCC GAC TTT

GCC ACC ACC ATC TGG CAG CAG ATG GAA GAA CTG

GGA ATG GCC CCT GCC CTG CAG CCC ACC CAG GGT

GCC ATG CCG GCC TTC GCC TCT GCT TTC CAG CGC

CGG GCA GGA GGG GTC CTA GTT GCC TCC CAT CTG

CAG AGC TTC CTG GAG GTG TCG TAC CGC GTT CTA

CGC CAC CTT GCC CAG CCC

8. The recombinant vector described under Patent Claim 3, in which the gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor encodes the nucleotide sequence shown below or a part of it:

ACC CCC CTG GGC CCT GCC AGC TCC CTG CCC CAG

AGC TTC CTG CTC AAG TGC TTA GAG CAA GTG AGG

AAG ATC CAG GGC GAT GGC GCA GCG CTC CAG GAG

AAG CTG TGT GCC ACC TAC AAG CTG TGC CAC CCC

GAG GAG CTG GTG CTG CTC GGA CAC TCT CTG GGC

ATC CCC TGG GCT CCC CTG AGC AGC TGC CCC AGC

CAG GCC CTG CAG CTG GCA GGC TGC TTG AGC CAA

CTC CAT AGC GGC CTT TTC CTC TAC CAG GGG CTC

CTG CAG GCC CTG GAA GGG ATC TCC CCC GAG TTG

GGT CCC ACC TTG GAC ACA CTG CAG CTG GAC GTC

GCC GAC TTT GCC ACC ACC ATC TGG CAG CAG ATG

GAA GAA CTG GGA ATG GCC CCT GCC CTG CAG CCC

ACC CAG GGT GCC ATG CCG GCC TTC GCC TCT GCT

TTC CAG CGC CGG GCA GGA GGG GTC CTA GTT GCC

TCC CAT CTG CAG AGC TTC CTG GAG GTG TCG TAC

CGC GTT CTA CGC CAC CTT GCC CAG CCC

9. The recombinant vector described under Patent Claim 3, in which the gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor has the nucleotide sequence shown in Fig. 4(A) or a part of it.

10. A transformant containing the recombinant vector containing a gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor.

11. The transformant described under Patent Claim 10, characterized by the fact that the gene is a DNA complementary to a messenger RNA which is obtained as a 15-

17S fraction by sucrose density gradient centrifugation and encodes the polypeptide having the activity of a human granulocyte colony-stimulating factor.

12. The transformant described under Patent Claim 10, in which the gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor encodes the polypeptide sequence shown below or a part of it:

Met Ala Gly Pro Ala Thr Gln Ser Pro Met Lys
Leu Met Ala Leu Gln Leu Leu Leu Trp His Ser
Ala Leu Trp Thr Val Gln Glu Ala Thr Pro Leu
Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu
Leu Lys Cys Leu Glu Gln Val Arg Lys Ile Gln
Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys
Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu
Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp
Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu
Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser
Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala
Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr
Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe
Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu
Gly Met Ala Pro Ala Leu Gln Pro Thr Gln Gly
Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg
Arg Ala Gly Gly Val Leu Val Ala Ser His Leu
Gln Ser Phe Leu Glu Val Ser Tyr Arg Val Leu
Arg His Leu Ala Gln Pro

13. The transformant described under Patent Claim 10, in which the gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor encodes the polypeptide sequence shown below or a part of it:

Thr Pro Leu Gly Pro Ala Ser Ser Leu Pro Gln
Ser Phe Leu Leu Lys Cys Leu Glu Gln Val Arg
Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu
Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro
Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly
Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln
Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu
Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu
Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val
Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met
Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro
Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala
Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr
Arg Val Leu Arg His Leu Ala Gln Pro

14. The transformant described under Patent Claim 10, in which the gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor encodes the nucleotide sequence shown below or a part of it:

ATG GCT GGA CCT GCC ACC CAG AGC CCC ATG AAG

CTG ATG GCC CTG CAG CTG CTG CTG TGG CAC AGT

GCA CTC TGG ACA GTG CAG GAA GCC ACC CCC CTG

GGC CCT GCC AGC TCC CTG CCC CAG AGC TTC CTG

CTC AAG TGC TTA GAG CAA GTG AGG AAG ATC CAG

GGC GAT GGC GCA GCG CTC CAG GAG AAG CTG TGT

GCC ACC TAC AAG CTG TGC CAC CCC GAG GAG CTG

GTG CTG CTC GGA CAC TCT CTG GGC ATC CCC TGG

GCT CCC CTG AGC AGC TGC CCC AGC CAG GCC CTG

CAG CTG GCA GGC TGC TTG AGC CAA CTC CAT AGC

GGC CTT TTC CTC TAC CAG GGG CTC CTG CAG GCC

CTG GAA GGG ATC TCC CCC GAG TTG GGT CCC ACC

TTG GAC ACA CTG CAG CTG GAC GTC GCC GAC TTT

GCC ACC ACC ATC TGG CAG CAG ATG GAA GAA CTG

GGA ATG GCC CCT GCC CTG CAG CCC ACC CAG GGT

GCC ATG CCG GCC TTC GCC TCT GCT TTC CAG CGC

CGG GCA GGA GGG GTC CTA GTT GCC TCC CAT CTG

CAG AGC TTC CTG GAG GTG TCG TAC CGC GTT CTA

CGC CAC CTT GCC CAG CCC

15. The transformant described under Patent Claim 10, in which the gene encoding the polypeptide having activity of human granulocyte colony-stimulating factor encodes the nucleotide sequence shown below or a part of it:

ACC CCC CTG GGC CCT GCC AGC TCC CTG CCC CAG

AGC TTC CTG CTC AAG TGC TTA GAG CAA GTG AGG

AAG ATC CAG GGC GAT GGC GCA GCG CTC CAG GAG

AAG CTG TGT GCG ACC TAC AAG CTG TGC CAC CCC

GAG GAG CTG GTG CTG CTC GGA CAC TCT CTG GGC

ATC CCC TGG GCT CCC CTG AGC AGC TGC CCC AGC

CAG GCC CTG CAG CTG GCA GGC TGC TTG AGC CAA

CTC CAT AGC GGC CTT TTC CTC TAC CAG GGG CTC

CTG CAG GCC CTG GAA GGG ATC TCC CCC GAG TTG

GGT CCC ACC TTG GAC ACA CTG CAG CTG GAC GTC

GCC GAC TTT GCC ACC ACC ATC TGG CAG CAG ATG

GAA GAA CTG GGA ATG GCC CCT GCC CTG CAG CCC

ACC CAG GGT GCC ATG CCG GCC TTC GCC TCT GCT

TTC CAG CGC CGG GCA GGA GGG GTC CTA GTT GCC

TCC CAT CTG CAG AGC TTC CTG GAG GTG TCG TAC

CGC GTT CTA CGC CAC CTT GCC CAG CCC

16. The transformant described under Patent Claim 10, in which the gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor has the nucleotide sequence shown in Fig. 4(A) or a part of it.

17. A polypeptide composition having the activity of a human granulocyte colony-stimulating factor, produced from the transformant containing the recombinant vector containing a gene encoding the polypeptide having the activity of a human granulocyte colony-stimulating factor.

18. A polypeptide having the activity of a human granulocyte colony-stimulating factor, represented by a part of the amino acid sequence shown in Fig. 4(B)(II).

3. Detailed Description

[Field in the Industry]

The present invention is related to a novel polypeptide, particularly to a polypeptide having the activity of a specific stimulating factor, mainly necessary to form human granulocyte colonies, that is, colony-stimulating factor (abbreviated as CSF in the following). In addition, it is related to a recombinant vector containing a gene encoding the polypeptide, a transformant containing the vector, and a CSF composition produced from the transformant.

[Prior Art]

Existence of factors promoting in vivo formation of colonies has been known through two-layer agar culture with bone marrow cells on the upper layer as target cells and with renal or fetal cells on the lower layer, leading to growth and differentiation of the upper layer cells to form colonies from neutrophilic granulocytes (called granulocytes in the following), monocytes or macrophages [Pluznik and Sach: J. Cell. Comp. Physiol., Vol. 66, p. 319 (1965); Bradley and Hetcalf: Aust. J. Exp. Biol. Med. Sci., Vol. 44, p. 287 (1966)].

The factors collectively called CSF are known to be produced from normally widely distributed cells such as T cells, monocytes, macrophages, fibroblasts, endothelial cells, etc. Among CSFs, there are subclasses, such as a granulocyte-monocyte/macrophage CSF (abbreviated as GM-CSF) which acts on the stem cells of granulocyte and monocyte/macrophage to stimulate their proliferation and induce their differentiation, forming colonies from granulocytes and monocytes/macrophages in agar, a monocyte/macrophage CSF (abbreviated as M-CSF) which mainly has the effect of forming colonies of monocytes/macrophages, a multi-CSF acting on relatively undifferentiated multiple potential progenitors, and a granulocyte CSF (abbreviated as G-CSF) which mainly forms granulocyte colonies as described in the present invention. It has been considered that the differentiation stages of target cells are different for the various subclasses [Asano: Metabolism and Disease, Vol. 22, p. 249 (1985); Yunis et al., "Growth and Maturation Factors", edited by Guroff, John Willey & Sons, NY, Vol. 1, p. 209 (1983)]. Accordingly, it is critically important for analysis of the hematopoietic mechanism and of the pathology of various hematological diseases, to purify each of the

subclasses to investigate their chemical properties and biological characteristics in detail. In particular, the biological effects of G-CSF, induction of the differentiation of bone marrow leukemia cells and enhancement of the functions of mature granulocytes, attracted significant attention. Clinical usefulness of G-CSF, particularly for the treatment and prevention of leukemia, is highly expected.

[Problems to be Solved by the Invention]

Traditionally, to isolate and purify G-CSF, cells were cultured and G-CSF was isolated from the culture supernatant. However, G-CSF is produced only at low concentrations. To obtain a small amount of G-CSF from a large quantity of culture liquid, a complicated process of purification is required. Due to this and other problems, purification of homogeneous G-CSF in a large quantity has not been achieved. Accordingly, large scale production of G-CSF using recombinant DNA technology has been desired.

[Means of Solving the Problems]

The present invention provides a polypeptide having the activity of a human granulocyte colony-stimulating factor and having the amino acid sequence described under Patent Claim 1. In addition, it provides a recombinant vector used for producing the polypeptide, a transformant obtained by transforming a host cell with the vector, and a G-CSF composition produced from the transformant.

A particularly important component of the present invention is a gene encoding the polypeptide having human G-CSF activity. Specifically, it is a DNA complementary to a messenger RNA (mRNA) which is obtained as a 15-17S faction by sucrose density gradient centrifugation and encodes the polypeptide having the activity of human G-CSF (cDNA). More specifically, it is the gene encoding the polypeptide I or II shown in Fig. 4(B) or a part of it. Even more specifically, it is the sequence corresponding to the nucleotide sequence from the ATG at positions 31-33 to the CCC at positions 640-642 or the nucleotide sequence from the ACC at positions 121-123 to the CCC at positions 640-642 from the 5'-end of the sequence shown in Fig. 4(A), the sequence shown in Fig. 4(A), or a part of it.

The gene of the present invention can be obtained by, for example, preparing the mRNA encoding G-CSF from mammalian cells capable of producing the polypeptide

with G-CSF activity, and then converting it to a double-stranded cDNA by standard methods.

The above mammalian cells as a source of the mRNA are the human oral cancer-derived cell line CHU-2 (Collection Nationale de Cultures de Microorganismes (C. N. C. M.) deposition No. I-483) in the present invention. Not only tumor cells, cells isolated from mammals or other cell lines also can be used. For the preparation of mRNA, by a method already used in the gene cloning for several physiologically active proteins, such as surface-active agent treatment and phenol treatment in the presence of a ribonuclease inhibitor (e.g., vanadium complex, etc.) [Berger and Birkenmeier: Biochemistry, Vol. 18, p. 5143 (1979)], or guanidine thiocyanate treatment followed by CsCl density gradient centrifugation [Chirgwin et al.: Biochemistry, Vol. 18, p. 5294 (1979)], total RNA is prepared, and then poly(A+)RNA (mRNA) can be obtained by affinity chromatography using oligo(dT)-cellulose or polyU-Sepharose on Sepharose 2B as carrier. Poly(A+)RNA also can be fractionated by sucrose density gradient centrifugation.

To confirm that the mRNA obtained above encodes the polypeptide having G-CSF activity, the mRNA is translated into protein. The physiological activity is investigated, or the protein is identified using an anti-G-CSF antibody. For example, the mRNA is injected into Xenopus laevis oocytes for translation [Gurdon et al.: Nature, Vol. 233, p. 177 (1972)]. Alternatively, a rabbit reticulocyte system or wheat germ system is used for the translation [Schleif and Wensink: "Practical Methods in Molecular Biology", Springer-Verlag, N. Y., (1981)].

For the identification of G-CSF activity, bone marrow cells are used and cultured on agar. Such methods have been reviewed [Metcalf: "Hemopoietic Colonies", Springer-Verlag, Berlin, Heidelberg, NY (1977)].

The mRNA obtained by a method described above is used as template to synthesize a single-stranded cDNA. From this single-stranded cDNA, a double-stranded cDNA is then synthesized, followed by making a recombinant plasmid with an appropriate vector DNA. The plasmid is transformed into Escherichia coli, etc., thereby obtaining a cDNA library.

To obtain a double-stranded cDNA from mRNA, for example, oligo(dT) complementary to the polyA chain on the 3'-end of mRNA is used as primer for reverse

transcriptase treatment. Alternatively, an oligonucleotide corresponding to a part of the amino acid sequence of G-CSF is synthesized, and then used as primer for reverse transcriptase treatment to synthesize a cDNA complementary to the mRNA. For obtaining a double-stranded cDNA, after alkaline treatment to degrade and remove mRNA, the resultant single-stranded cDNA is treated with reverse transcriptase or DNA polymerase (e.g., Klenow fragment, etc.) followed by treatment with SI nuclease, etc. Alternatively, it can be obtained by direct treatment with RNase H or DNA polymerase (e.g. DNA polymerase I from E. coli, etc.) [for example, Maniatis et al.: Molecular Cloning, Cold Spring Harbor Laboratory (1982); and Gubler and Hoffman: Gene, Vol. 25, p. 263 (1983)].

The double-stranded cDNA thus obtained is recombinated into an appropriate vector, such as an EK-type plasmid vector (e.g., pSC101, pDF41, ColE1, pMB9, pBR322, pBR327, pACYC1, etc.) or a phage vector (e.g., λgt, λC, λgt10, λgtWES, etc.), followed by transformation into E. coli (x1776, HB101, DH1, C600 strain, etc.), etc., thereby obtaining a cDNA library (for example, see the above "Molecular Cloning").

For ligating the double-stranded cDNA with the vector, an appropriate, chemically synthesized DNA fragment is added to attach a ligatable end to the end of the DNA, and then it is treated along with the vector DNA, that has been opened in advance with a restriction enzyme, with T4 phage DNA ligase in the presence of ATP. Alternatively, dG and dC (or dA and dT) chains are added to the vector DNA, that has been opened in advance with a restriction enzyme, and the double-stranded cDNA, and then, for example, a solution containing the two DNAs is gradually cooled (see the above "Molecular Cloning").

To transform the recombinant DNA thus obtained into a host cell, for example, with E. coli as the host cell, the method described by Hanahan in detail can be used [J. Mol. Biol.: Vol. 166, p. 557 (1983)]. Thus, the recombinant DNA is added to competent cells prepared in the presence of $CaCl_2$, $MgCl_2$ or RbCl for transformation.

To identify cells containing the target gene, one of the following methods can be used: the plus-minus method used for cloning interferon cDNA [Taniguchi et al.: Proc. Jpn. Acad., Vol. 55, Ser. B, p. 464 (1979)], the hybridization-translation assay method [Nagata et al.: Nature, Vol. 284, p. 316 (1980)], the colony or plaque hybridization method using an oligonucleotide chemically synthesized based on the amino acid

sequence of the protein as a probe [Wallace et al.: Nucleic Acids Res., Vol. 9, p. 879 (1981)], etc.

The cloned fragment containing the gene encoding a polypeptide with the activity of human G-CSF thus obtained is recombinated into an appropriate vector DNA again, and accordingly it can be transformed into an eukaryotic or prokaryotic host cell. By introducing an appropriate promoter and a sequence(s) for expression, the gene can be expressed in the host cell.

The prokaryotic host cells, for example, include Escherichia coli, Bacillus subtilis, Bacillus thermophilus, etc. To express the target gene in such a host cell, it is transformed into the host cell in a plasmid vector containing replicons derived from a strain compatible with the host cell, that is, transcription initiation and regulation sequences. Moreover, it is desirable that the vector contains a selection marker for the expression in a host cell.

For example, E. coli can be transformed with the vector pBR322, being the host cell of the vector [Boliver et al.: Gene, Vol. 2, p. 95 (1975)]. The vector pBR322 contains ampicillin- and tetracycline-resistant genes. Utilizing the resistance to either antibiotic, the transformants can be identified. The promoters necessary for gene expression in the prokaryotic host cells include β-lactamase gene promoter [Chang et al.: Nature, Vol. 275, p. 615 (1978)], lactose promoter [Goeddel et al.: Nature, Vol. 281, p. 544 (1979)] and tryptophan promoter [Goeddel et al.: Nucleic Acids Res., Vol. 8, p. 4057 (1980)]. Each of the promoters can be used to express the polypeptide with the activity of human G-CSF of the present invention.

To obtain the polypeptide with the activity of human G-CSF using the host-vector system as described above, the recombinant DNA with the gene inserted at an appropriate position of the above vector is transformed into the host cell, followed by culturing of resultant transformant. For further isolation and purification of the polypeptide from the cells or culture supernatant, known procedures can be used.

In general, eukaryotic genes have polymorphism as exemplified by interferon gene [Nishi et al.: J. Biochem. Vol. 97, p. 153 (1985)]. Due to polymorphism one or more amino acid residues can be different. There also could be changes of nucleotide sequence without any changes of amino acid residue.

A polypeptide with a deletion or addition of one or more amino acid residues or with one or more amino acid residues substituted by one or more amino acid residues in the amino acid sequence shown in Fig. 4(B) could still have the activity of human G-CSF. For example, it is well-known that a polypeptide obtained by converting the nucleotide sequence corresponding to cysteine to a nucleotide sequence corresponding to serine in the gene for human interleukin-2 (IL-2) still maintains the activity of interleukin-2 [Wang et al.: Science, Vol. 224, p. 1431 (1984)]. Accordingly, for all polypeptides, native or artificially synthesized, as long as they have the activity of human G-CSF, their genes are covered by the present invention.

The polypeptide with the activity of human G-CSF in the present invention, the recombinant vector containing the gene encoding the polypeptide, the transformant containing the vector, and the method for obtaining the expressed human G-CSF composition are described as follows.

(1) Preparation of probe

The N-terminal amino acid sequence of human G-CSF purified to homogeneity from the culture supernatant of the tumor cell line CHU-2 was determined. In addition, by bromocyan digestion and trypsin treatment, fragments are prepared and their amino acid sequences are also determined [Practical Example 3 (i) (ii) (iii)].

From the amino acid sequences, three nucleotides, probe (A), probe (LC) and probe (IWQ) corresponding to the sequence shown in Fig. 1 were synthesized. (Practical Example 4) probe (A) is a mixed type probe comprising 14 nucleotides.

Probe (IWQ) comprises 30 nucleotides using deoxyinosine as used in the gene cloning of human cholecystokinin [Takanishi et al.: Proc. Natl. Acad. Sci. USA, Vol. 82, p. 1931 (1985)]. Probe (LC) is a probe comprising 24 nucleotides synthesized based on the nucleotide sequence shown in Fig. 3, corresponding to the amino acid sequence from No. 32 to No. 39 from the N-terminus shown in Practical Example 3 (i).

Chemical synthesis of nucleotide can be performed by the modified phosphotriester method used as a solid phase method, as reviewed by Marang [Tetrahedron, Vol. 39, p. 3-22 (1983)], using a commercially available automated synthesis apparatus (e.g. the one from Applied Biosystems).

The probes used can be based on amino acid sequences at positions other than those for the probes used in the present invention.

(2) Construction of cDNA library

A guanidine thiocyanate solution is added to CHU-2 cells for homogenization. Total RNA is obtained by CsCl density gradient centrifugation method.

From the total RNA, poly(A+) RNA is isolated using an oligo(dT) cellulose column. Single-stranded cDNA is synthesized with reverse transcriptase. RNase H and E. coli DNA polymerase are added to obtain double-stranded cDNA. A dC chain is added to the resultant double-stranded cDNA, and the cDNA is connected with pBR322 vector with a dG chain added to the PstI digestion site. By transformation into E. coli strain X1776, a pBR322-based cDNA library is obtained (Practical Examples 5 and 6).

Similarly, a EcoRI linker is used to connect the double-stranded cDNA with λgt10 vector to construct a λ phage-based cDNA library (Practical Example 7).

(3) Screening

Clones of the pBR322-based cDNA library are fixed on Whatman No. 541 filter paper, and a 32P-labeled probe (IWQ) is used to perform colony hybridization, thereby selecting one clone. This clone is further investigated by Southern blotting method [Southern: J. Mol. Biol. Vol. 98, p. 503 (1975)], and it is found that it also hybridizes with probe (A).

The nucleotide sequence of this clone is determined by the dideoxy method [Sanger: Science, Vol. 214, p. 1205 (1981)].

The resultant nucleotide sequence of the cDNA insert is shown in Fig. 2. As shown in Fig. 2, this cDNA insert comprises 308 nucleotides including probe (IWQ) and probe (A). It is thus known that it has an open reading frame encoding an 83-amino-acid sequence shown in Practical Example 3 (iii).

The plasmid derived from pBR322 containing the 308 base pairs is abbreviated as pHCS-1 (Practical Example 8).

A DNA fragment containing the 308 base pairs from pHCS-1 is radiolabeled by the nick translation method (see above mentioned "Molecular Cloning"), and used as a probe to perform screening gt10 by plaque hybridization [Benton and Davis: Science, Vol. 196, p. 180 (1977)] with the λ phage-based cDNA library, thereby selecting 5 clones. For clones considered to contain the cDNA, their nucleotide sequences are determined as above (Fig. 3).

As shown in Fig. 3, this cDNA insert has a large open reading frame.

The amino acid sequence encoded by this cDNA can be represented as shown in Fig. 3.

The pBR322-maintaining Escherichia coli (E. coli) strain X1776 with the cDNA inserted at the EcoRI digestion site is deposited with the Academy of Industrial Technologies, Institute for Microbiological Industrial Technologies (FERM P-8352). A plasmid with this cDNA inserted into pBR327 [Soberon et al.: Gene, Vol. 9, p. 287 (1980)] at the EcoRI site is called pBRG4.

The pBRG4 thus obtained is digested with the restriction enzyme EcoRI to obtain a DNA fragment including a cDNA of about 1500 base pairs. The fragment is radiolabeled by the nick translation method (see above mentioned "Molecular Cloning"), and used as a probe to perform screening gt10 by plaque hybridization (see above mentioned Benton and Davis's reference) with the λ phage-based cDNA library again. At this time, two nitrocellulose filter papers with the λ phage DNA immobilized are prepared, and plaque hybridization with probe (LC) is similarly performed. Phages positive with both probes are selected, and clones considered to represent full-length are selected. Using the dideoxy method, the nucleotide sequences of the cDNA inserts are determined, and the results are shown in Fig. 4(A).

This cDNA has a large open reading frame, and encodes an amino acid sequence as shown in Fig. 4(A).

Comparison with the N-terminal amino acid sequence of G-CSF protein shown in Fig. 3(i) reveals that this cDNA contains a nucleotide sequence corresponding to a signal peptide encoded by 90 base pairs, starting at the ATG sequence at nucleotide positions 31-33 from the 5'-end and ending at the GCC sequence at positions 118-120, and a

mature G-CSF polypeptide encoded by 522 base pairs, starting at the ACC sequence at positions 121-123 and ending at the CCC sequence at positions 640-642. Thus, the polypeptide of amino acid sequence I shown in Fig. 4(B) comprises 204 amino acid residues, with a molecular weight of 21977.35 dalton. Similarly, the polypeptide of amino acid sequence II comprises 174 amino acid residues, with a molecular weight of 18671.42 dalton (Practical Example 10).

As an initiation site for the protein, in addition to the positions 31-33, ATGs at 58-60 and 67-69 are also possible.

The pBR327-maintaining Escherichia coli (E. coli) strain X1776 with the cDNA inserted at the EcoRI digestion site is deposited with the Academy of Industrial Technologies, Institute for Microbiological Industrial Technologies (FERM P-8453).

Fig. 5 shows restriction enzyme digestion sites of the resultant gene.

(4) Construction of recombinant vector

From the pBRV2 plasmid thus obtained (Practical Example 10), a cDNA fragment for G-CSF polypeptide is cut out with the restriction enzymes, and undergoes one of the following procedures.

(1) It is ligated with a synthetic linker annealed with a fragment prepared from pKK223-3 (Pharmacia) containing a tac promoter, thereby constructing a recombinant vector (Practical Example 11).

(2) It is ligated with a synthetic linker annealed with three fragments prepared from pPL-lambda (Pharmacia) containing P_L promoter, thereby constructing a recombinant vector (Practical Example 12).

(3) It is ligated with a synthetic linker annealed with a fragment prepared from pOYI plasmid containing trp promoter, thereby constructing a recombinant vector (Practical Example 13).

(5) Preparation of transformant, and culture and expression

The above three recombinant vectors are transformed into E. coli strain DH1, E. coli strain N4830 or E. coli strain JM105 by the calcium chloride method or rubidium

chloride method described in the above mentioned Molecular Cloning (Practical Examples 11, 12 and 13).

The resulting transformant is cultured in Luria medium containing ampicillin for expression. If necessary, induction is performed (Practical Example 14).

(6) Recovery, purification and amino acid analysis of E. coli-derived G-CSF polypeptide

The supernatant of the transformant culture is subjected to centrifugation to collect the bacteria. The cells are lysed by lysozyme treatment and repeated freeze and thaw cycles. After guanidine chloride treatment, centrifugation is performed to obtain a supernatant.

The supernatant is applied to an Ultrogel ACA54 column (LKB) for gel filtration. Active fractions were concentrated by filtration.

Subsequently, an aqueous solution of trifluoroacetic acid containing n-propanol is added. After having been allowed to stand on ice, centrifugation is performed, followed by adsorption and elution procedures on a reverse phase C18 column. The activity was assayed in each fraction after elution, and the activity peak is collected and freeze-dried. The freeze-dried powder is dissolved and applied to high performance liquid chromatography. The same purification procedures as above are repeated. The resultant polypeptide is applied to SDS-polyacrylamide gel electrophoresis to confirm target G-CSF polypeptide as a single band (Practical Example 15).

The polypeptide thus obtained shows the activity of human G-CSF (Practical Example 16).

Furthermore, for amino acid analysis, the amino acid composition of the purified G-CSF polypeptide is obtained with a Hitachi 835 Automated Amino Acid Analyzer (Hitachi Production) by the special amino acid analysis method. Moreover, N-terminal amino acid analysis is performed with a gas-phase sequencer by Edman degradation and high performance liquid chromatography on Ultrasphere-ODS column (Practical Example 17).

[Practical Examples]

In the following, practical examples are used to further describe the present invention in detail. First, assay for CSF activity is described as a reference example.

<Reference Example> Assay for CSF activity

In the present invention, assay for CSF activity (abbreviated as CSA in the following) is performed as follows.

[Assay for CSA]

(a) Use of human bone marrow cells

It is performed by the single layer soft agar culture method in accordance with Bradley T. R., Metcalf D. et al.'s method [Aust. J. Exp. Biol. Med. Sci. Vol. 44, p. 287-300 (1966)]. Thus, 0.2 mL of bovine fetal serum, 0.1 mL of test sample, 0.1 mL of non-adherent human bone marrow cell suspension (1-2 x 10^5 nucleated cells), 0.2 mL of modified McCoy's 5A culture medium, and 0.4 mL of modified McCoy's 5A culture medium containing 0.75% agar are mixed and transferred into a plastic tissue culture dish with a diameter of 35 mm for solidification, followed by culture at 37°C under conditions of 5% carbon dioxide/95% air and 100% humidity. After 10 days, the formed colonies are counted (one collection of at least 50 cells is counted as one colony). Activity forming one colony is defined as one unit, thereby calculating the CSA.

(b) Use of mouse bone marrow cells

0.4 mL of horse serum, 0.1 mL of test sample, 0.1 mL of C3H/Hc (female) mouse bone marrow cell suspension (0.5-1 x 10^5 nucleated cells), and 0.4 mL of modified McCoy's 5A culture medium containing 0.75% agar are mixed and transferred into a plastic tissue culture dish with a diameter of 35 mm for solidification, followed by culture at 37°C under conditions of 5% carbon dioxide/95% air and 100% humidity for 5 days. Formed colonies are counted (one collection of at least 50 cells is counted as one colony). Activity forming one colony is defined as one unit, thereby calculating CSA.

The modified McCoy's 5A culture medium used in above methods (a) and (b) and the non-adherent human bone marrow cell suspension used in above method (a) are prepared as follows.

[Modified McCoy's 5A culture medium (2x concentration)]

12 g of McCoy's 5A culture medium (GIBCO), 2.55 g of MEM amino acid vitamin medium (Nissui Pharmaceuticals), 2.18 g of sodium bicarbonate and 50000 units of potassium penicillin G are dissolved in 500 mL of double-distilled water, followed by filtration through 0.22 μm Millipore filter.

[Non-adherent human bone marrow cell suspension]

Bone marrow fluid from healthy donors by chest bone puncture is diluted 5 times with RPMI 1640 culture medium, and then layered onto Ficol-Paque liquid (Pharmacia). After centrifugation at 400 x g for 30 min at 25°C, cells at the interface are recovered (specific gravity <1.077). The cells are washed, and suspended in RPMI 1640 medium containing 20% fetal bovine serum at a density of 5 x 10^6 cells/mL, followed by transfer to a 25 cm^2 plastic tissue culture flask. After culture in a carbon dioxide incubator for 30 min, non-adherent cells in the supernatant are recovered, and transferred to a 25 cm^2 plastic tissue culture flask again. After culture for 2 hr and 30 min, non-adherent cells in the supernatant are collected for use.

Practical Example 1: Establishment of CHU-2

The tumor of an oral cancer patient with a significant neutrophilia was transplanted into a nu/nu mouse. This tumor showed significant growth and an increased neutrophil count at about 10 days after transplantation. The tumor was isolated under sterile condition at 12 days after transplantation, and cut into 1-2 mm^3 cubes, followed by culture as follows.

10-15 pieces of the tumor cut above were placed in a 50 mL plastic centrifuge tube, and 5 mL of a trypsin solution (containing 0.25% trypsin and 0.02% EDTA) was added. After 10 min shaking in a 37°C water bath, the supernatant was discarded. Again, 5 mL of the same trypsin solution was added, and trypsin treatment was performed at 37°C for 15 min with agitation. The cell suspension was recovered as the supernatant, and 1 mL of bovine fetal serum was added to stop the action of trypsin, followed by storage on ice.

The above procedures were repeated to recover the cell suspension, which was then combined with one obtained previously. By centrifugation at 1,500 rpm for 10 min, a cell pellet was obtained. The cell pellet was washed twice with F-10 containing 10% fetal bovine serum, and then seeded in a 25 cm^2 plastic tissue culture flask at a

concentration of 5 x 10^6 cells/flask. F-10 culture medium containing 10% fetal bovine serum was used. After overnight incubation in a carbon dioxide incubator (carbon dioxide concentration: 5%; humidity: 100%), the supernatant and non-adherent cells were removed. Fresh culture medium was added, and the culture was continued. On day 6 from start of the culture, the cells grew into confluence. At that point the culture medium was replaced with a fresh one. On the next day, the culture medium was discarded, and 2 mL of anti-mouse erythrocyte antibody (Cappel) diluted 5-fold with RPMI 1640 and 2 mL of guinea pig complement (Kyokuto Pharmaceuticals) diluted 2.5-fold with the same RPMI 1640 were added, followed by culture at 37°C for 20 min. After incubation, nu/nu mouse-derived fibroblasts were removed by washing twice with F-10 containing 10% fetal bovine serum. F-10 culture medium containing 10% fetal bovine serum was added, followed by further culture for 2 days. An aliquot of the cells was removed for cloning by the limiting dilution method.

Eleven clones were obtained, and their CSF activity was investigated. A clone with an about 10-fold higher activity than others was obtained (CHU-2).

Practical Example 2: Isolation of CSF

Cells were collected from two 150 cm^3 culture flasks with the cells established as above grown into complete confluence. The cells were suspended in 500 mL of F-10 culture medium containing 10% fetal bovine serum, and then transferred to a 1580 cm^3 glass roller bottle (Belco), followed by rotary culture at a speed of 0.5 rpm. When the cells grew to complete confluence on the inner wall of the roller bottle, the culture medium was replaced with serum-free RPMI 1640. After culture for 4 days, the culture supernatant was collected. F-10 culture medium containing 10% fetal bovine serum was added, and the culture was continued. After culture for 3 days, the culture medium was replaced again with serum-free RPMI 1640. After culture for 4 days, the culture supernatant was collected. By repeating the same procedures, every week from one bottle, 500 mL of serum-free culture medium was obtained. Moreover, by this method, the cells could be maintained over a fairly long period of time, and the culture supernatant could be collected.

To 5 L of the resultant culture supernatant as one batch, Tween 20 was added to 0.01%. After concentration about 1000-fold by ultrafiltration using Hollow Fiber DC-4 and Amicon PM-10 (Acon), purification was performed by the following procedures.

(i) An Ultrogel ACA 54 column (LKB) with a diameter of 4.6 cm and a length of 90 cm was used. Gel filtration of 5 mL of the above culture supernatant was performed with 0.01 M Tris-hydrochloric acid buffer (pH 7.4) containing 0.15 M NaCl and 0.01% Tween 20 (Kanai Chemicals) at a flow rate of about 50 mL/hr. The column was subjected to calibration in advance with bovine serum albumin (molecular weight 67,000), ovalbumin (molecular weight 45,000), and cytochrome C (molecular weight 12,400). After gel filtration, 0.1 mL of each fraction was used, after 10-fold dilution, for activity assay as described above in "CSA assay method (b)". The result revealed that there was a macrophage-preferred CSA in fractions of Ve = 400-700 mL, and a granulocyte-preferred CSA in fractions of Ve = 800-1200 mL. The later fractions were pooled, and concentrated to about 5 mL by ultrafiltration using PM-10 (Amicon).

(ii) To the above concentrated sample, 0.1% trifluoroacetic acid solution containing 30% n-propanol (Tokyo Kassei, amino acid sequencing grade) was added. After being allowed to stand on ice for 15 min, centrifugation was performed at 15,000 rpm for 10 min to remove precipitate. Subsequently, after application to a μ Bondapak C18 column (Waters, for semi-preparation, 8 mm x 30 cm) that had been equilibrated with the above n-propanol trifluoroacetic acid solution, elution was performed with a 30-60% linear concentration gradient of n-propanol-containing 0.1% trifluoroacetic acid solution. The high performance liquid chromatography apparatus was Hitachi model 685-50, with the detector being Hitachi model 638-41 detector (both from Hitachi Production). Absorption at both 220 nm and 280 nm was measured. After elution, 10 μL of each fraction was used, after 100-fold dilution, for activity assay as described above in "CSA assay method (b)". The result revealed that there was a peak which was eluted at 40% of n-propanol. The peak was pooled and re-chromatographed under the same conditions, followed by CSA assay in the same way. Again, the activity peak was observed at 40% of n-propanol. This peak was pooled (4 fractions = 4 mL) and freeze-dried.

(iii) The above freeze-dried powder was dissolved in 200 μL of 0.1% trifluoroacetic acid solution containing 40% n-propanol, and then applied to high performance liquid chromatography (HPLC) using a TSK-G3000SW column (Toyo Sotatsu, 7.5 mm x 60 cm). Elution was performed with the same solution at 0.4 mL/min, and fractions of 0.4 mL each were collected with a fraction collector of FRAC-100 (Pharmacia). The fractions were investigated for CSA as above. The activity was observed in a fraction with a retention time of 37-38 min (molecular weight is

corresponding to about 20,000). This fraction was recovered, and further purified on an analytical μ Bondapak C18 column (4.6 mm x 30 cm). The main peak was recovered and freeze-dried. The resultant sample was evaluated by above "CSA assay method (a)", and it was confirmed to have the activity of human G-CSF.

Practical Example 3:

(i) Determination of N-terminal amino acid sequence

Using a gas phase sequencer (Applied Biosystems), the test sample was subjected to (Edman) degradation, and then the resultant PTH amino acid was analyzed by a standard method using a high performance liquid chromatography apparatus (Beckman Instruments) and Ultrophere-ODS column (Beckman Instruments). The column (5 μm, diameter 4.6 mm, length 250 cm) was equilibrated with a starting buffer (15 mM sodium acetate buffer, pH 4.5, containing 40% acetonitrile), and then the test sample (dissolved in 20 μL of the starting buffer) was applied. Isocratic elution was performed with the starting buffer at a flow rate of 1.4 mL/min. Column temperature was maintained at 40°C. Detection of PTH amino acid was performed based on ultraviolet absorption at 269 nm and 320 nm. Standard PTH amino acids (Sigma), 2 nmol each, were separated in advance by the same system to determine their retention times. From the retention time of test sample, it could be identified. The results revealed the amino acid sequence from the N-terminus to No. 40 residue was as follows.

H_2N-Thr-Pro-Leu-Gly-
Pro-Ala-Ser-Ser-Leu-
(10)
Pro-Gln-Ser-Phe-Leu-
Leu-Lys-Cys-Leu-Glu-
(20)
Gln-Val-Arg-Lys-Ile-
Gln-Gly-Asp-Gly-Ala-
(30)
Ala-Leu-Gln-Glu-Lys-
Leu-Cys-Ala-Thr-Tyr-
(40)
Lys-

(ii) Bromocyan degradation

The test sample was dissolved in 70% formic acid, and 200 equivalent of sublimation-purified bromocyan was added, followed by overnight reaction at 37°C. The reaction mixture was freeze-dried, and fractionated by HPLC on a TSK G3000SW column (Toyo Sotatsu), thereby obtaining four peaks, named as CN-1, CN-2, CN-3 and CN-4 in the order of molecular weights being from large to small. For CN-1 and CN-2, the yields of that were higher, they were analyzed using a gas-phase sequencer (Applied Biosystems) under the same conditions as in (i).

The results revealed CN-1 was a peptide from the N-terminus of G-CSF, and that CN-2 had the following amino acid sequence.

Pro-Ala-Phe-Ala-Ser-
Ala-Phe-Gln-Arg-Arg-
Ala-Gly-Gly-Val-Leu-
Val-Ala-Ser-His-Leu-
Gln-

(iii) Trypsin degradation

The test sample was dissolved in 0.1 M Tris-hydrochloric acid buffer (pH 7.4) containing 8M urea, and then 0.1 M Tris-hydrochloric acid buffer (pH 7.4) containing 0.1% 2-mercaptoethanol was added so that the final concentration of urea was 2 M. Subsequently, TPCK-treated trypsin (Sigma) was added so that the ratio of test sample: enzyme was 50:1. After reaction at 4°C for 4 hr, the same amount of TPCK-treated trypsin was further added, followed by further reaction at 25°C for 16 hr. After reaction, the reaction mixture was applied to reverse phase high performance liquid chromatography using a C8 column (Yamamura Chemical). For elution, n-propanol containing 0.1% TFA was used and the concentration of n-propanol was increased linearly between 5% and 60%. Among peaks detected by ultraviolet absorption at 280 nm, the main peak was subjected to amino acid sequencing using a gas phase sequencer (Applied Biosystems) under the same conditions as in (i). The results revealed that the main peak contained the following sequence, which contained a portion of CN-2 of (ii).

Gln-Leu-Asp-Val-Ala-
Asp-Phe-Ala-Thr-Thr-
Ile-Trp-Gln-Gln-Met-
Glu-Glu-Leu-Gly-Met-
Ala-Pro-Ala-Leu-Gln-
Pro-Thr-Gln-Gly-Ala-
Met-Pro-Ala-Phe-Ala-
Ser-

Practical Example 4: Preparation of DNA probe

(i) Synthesis of probe (IWQ)

Based on the 10 amino acid residue sequence

5' Ile-Trp-Gln-Gln-Met-
Glu-Glu-Leu-Gly-Met

among the amino acid sequences obtained in Practical Example 3 (iii), a 30-mer nucleotide sequence was obtained (Fig. 1). In the sequence shown in Fig. 1, for example, at the No. 9 position from 5'-end, it is an equal amount mixture of dA and dG. The raw material nucleotides used were mainly dimers. If necessary, mononucleotides also can be used. 20 mg of starting material nucleotide resin Ap-d(G) (Yamasa Soybean Sauce) was applied to a column equipped with glass filter, and thoroughly washed with methylene chloride. The 4, 4'-dimethoxytrityl group was removed with 3% trichloroacetic acid-containing methylene chloride solution. Subsequently, the column was washed several times with 1 mL of methylene chloride, and then washed with anhydrous pyridine to replace the solvent. 20 mg of nucleotide dimer (DMTr)ApTp(NHR_3) (Nippon Zeon; NHR_3 is triethylammonium, while DMTr is dimethoxytrityl) and 0.2 mL pyridine were added. The inside of the column was vacuum-dried with a vacuum pump. 20 mg of 2, 4, 6-trimethylbenzene-sulfonyl-3-nitrotriazolide (MSNT, Wako Pure Chemicals) and 0.2 mL of anhydrous pyridine were added. The inside of the column was replaced with nitrogen gas. By shaking at room temperature for 45 min, the nucleotide resin and dimer were condensed.

After the reaction, the column was washed with pyridine, and then the unreacted OH group was acetylated with an excessive amount of anhydrous acetic acid-4-dimethyl-aminopyridine. The column was washed with pyridine again. Subsequently, the same procedures were repeated to condense the followings in this particular order: (DMTr)Ip(NHR_3), (DMTr)GpGp(NHR_3), (DMTr)Ip(NHR_3), equal amount mixture of (DMTr)CpTp(NHR_3) and (DMTr)TpTp(NHR_3), equal amount mixture of (DMTr)ApAp(NHR_3) and (DMTr)ApGp(NHR_3), equal amount mixture of (DMTr)ApGp(NHR_3) and (DMTr)GpGp(NHR_3), (DMTr)GpAp(NHR_3), (DMTr)TpGp(NHR_3), equal amount mixture of (DMTr)ApAp(NHR_3) and (DMTr)GpAp(NHR_3), (DMTr)CpAp(NHR_3), equal amount mixture of (DMTr)ApAp(NHR_3) and (DMTr)ApGp(NHR_3), (DMTr)GpCp(NHR_3), (DMTr)TpGp(NHR_3), (DMTr)Ip(NHR_3) and (DMTr)ApTp(NHR_3) [(DMTr)Ip(NHR_3) was from Yamasa Soybean Sauce, while others were from Nippon Zeon]. After the reaction in the final step, without acetylation, the resin was washed with pyridine, methylene chloride and ether, in this particular order, followed by drying. The dried resin was suspended in 1.7 mL of a mixture of 1 mL of dioxane containing 1 M tetramethylguanidine and 1 M α-picolinic aldoxime, 0.5 mL of pyridine and 0.2 mL of water. After allowing it to stand at room temperature overnight, it was concentrated under reduced pressure to 100-200 μL. A small amount of pyridine (2-3 drops) was added to the concentrate and then 2-3 mL of concentrated ammonia water was added, followed by heating at 55°C for 6 hr. Ethyl acetate was added for extraction and separation. The resultant aqueous phase was concentrated under reduced pressure, and then dissolved in 50 mM triethylammonium acetate solution (pH 7.0), followed by column chromatography using a C-18 column (1.0 x 15 cm, Waters). Elution was performed with a 10%-30% linear concentration gradient of acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.0). A peak eluted at an acetonitrile concentration of around 25% was concentrated under reduced pressure.

To the concentrate, 80% acetic acid solution was added, and the mixture was allowed to stand at room temperature for 30 min. Ethyl acetate was added for extraction and separation. The aqueous phase was concentrated under reduced pressure. The resultant concentrate was applied to high performance liquid chromatography using a C-18 column (Sensyu Scientific, SSC-ODS-272, 6 ø x 200 mm) for further purification. Elution was performed with a 10%-20% linear concentration gradient of acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.0). The synthetic DNA was obtained at a yield of at least 10 A260 units.

The sequence of the resultant oligonucleotide was determined by the Maxam-Gilbert method [Meth. Enzym., Vol. 65, p. 499 (1980)], and it was confirmed to have the sequence shown in Fig. 1.

(ii) Synthesis of probe (A)

Based on the 5 amino acid residue sequence

5Met-Pro-Ala-Phe-Ala among the amino acid sequences obtained in Practical Example 3 (iii), a 14-mer nucleotide sequence was obtained (Fig. 1).

The synthesis was performed by the same method as with probe (IWQ). To nucleotide resin AP-d(T) (Yamasa Soybean Sauce), (DMTr)CpAp(NHR_3), (DMTr)GpGp(NHR_3), (DMTr)CpAp(NHR_3), (DMTr)CpTp(NHR_3), equal amount mixture of (DMTr)CpGp(NHR_3) and (DMTr)CpCp(NHR_3), (DMTr)ApGp(NHR_3), (DMTr)TpGp(NHR_3), equal amount mixture of (DMTr)GpGp(NHR_3) and (DMTr)CpGp(NHR_3), (DMTr)ApAp(NHR_3), and equal amount mixture of (DMTr)CpAp(NHR_3) and (DMTr)CpGp(NHR_3) and (DMTr)GP(NHR_3) (all from Nippon Zeon) were condensed sequentially, in this particular order, thereby the synthetic DNA was obtained at a yield of at least 10 A260 units. The sequence of the resultant oligonucleotide was determined by the Maxam-Gilbert method, and it was confirmed to have the sequence shown in Fig. 1.

(iii) Synthesis of probe (LC)

Automated synthesis was performed using DNA synthesizer model 380A from Applied Biosystems. This method was based on the principles described by Caruthers et al. [J. Am. Chem. Soc. Vol. 103, p. 3185 (1981)], and is called the phosphoamidite method.

The phosphoamidite form of (DMTr)-dT activated with tetrazol in advance was condensed to dG-S (S is a carrier) with the 5'-dimethoxytrityl group (DMTr) de-protected. Unreacted hydroxyl group was acetylated, and then in the presence of water iodic acid treatment was performed to introduce a phosphate group. The DMTr group was de-protected, and then the same procedures were repeated to synthesize the 24-mer nucleotide with the sequence shown in Fig. 1. The resultant nucleotide was cleaved from

the carrier and de-protected, followed by purification by reverse phase high performance liquid chromatography using a C18 column (Sensyu Scientific, SSC-ODS-272).

Practical Example 5: Culture of CHU-2 cells and purification of mRNA

1) Culture of CHU-2 cells and recovery of cells

The established CHU-2 cells were grown to complete confluence in two 150 cm^3 culture flasks. The cells were suspended in 500 mL of RPMI 1640 culture medium containing 10% fetal bovine serum, and then transferred to a 1580 cm^3 glass roller bottle (Belco). After rotary culture for 4 days at a speed of 0.5 rpm, when the cells grew into complete confluence on the inside wall of the roller bottle, the culture medium was discarded from the roller bottle. 100 mL of physiological saline containing 0.02% EDTA that had been heated to 37°C in advance was added. After heating at 37°C for 2 min, the cells were removed from the wall by pipetting. The resultant cell suspension was centrifuged at 1500 rpm for 10 min to obtain a cell pellet. The cells were suspended in 5 mL of physiological saline without containing EDTA, and centrifuged at 1500 rpm for 10 min to obtain a cell pellet again (wet weight about 0.8 g). The cells thus obtained were stored at -80°C until RNA isolation.

2) Purification of mRNA

mRNA isolation from the CHU-2 cells obtained above was essentially performed as described in Molecular Cloning [Maniatis et al.: Cold Spring Harbor, p. 196 (1982)]. The CHU-2 cells stored frozen (wet weight 3.8 g) were suspended in 20 mL of 6 M guanidine solution (6 M guanidine cyanate, 5 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, and 0.5% sodium sarcosylsulfate), and thoroughly mixed for 2-3 min with a vortex mixer. In a 20 mL syringe equipped with a 18 G injection needle, the mixture was aspirated and discharged 10 times. 6 mL of 5.7 M CsCl - 0.1 M EDTA (pH 7.5) was first placed on a polyallomer [phonetic] centrifuge tube that fits into the Beckman SW40Ti rotor, and then about 6 mL of the above viscous cell homogenate in guanidine solution was introduced so that the tube was full. Four tubes such prepared were centrifuged at 30,000 rpm for 15 hr at 20°C. The resultant pellet was washed three times with a small amount of 70% ethanol.

The pellets from all tubes were pooled and dissolved in 550 μL of water. The NaCl concentration was adjusted to 0.2 M. After phenol-chloroform (1:1) treatment and chloroform treatment, 2 volumes of ethanol were added to perform ethanol precipitation,

thereby obtaining total RNA (from 3.8 g of wet cells about 10.1 mg of total RNA was obtained). Poly(A+) RNA purification from total RNA was performed as follows. The method was affinity purification, based on the fact that on the 3'-end of mRNA there is a polyA chain. Oligo(dT)-cellulose (P-L Biochemicals, Type 7) was used. The total RNA was dissolved in an adsorption buffer (10 mM Tris-hydrochloric acid (pH 7.5) containing 0.5 M NaCl, 1 mM EDTA, and 0.1% SDS). After heating at 65°C for 5 min, the solution was applied onto the oligo(dT)-cellulose column that had been filled with the same buffer. Elution was performed with TE buffer (10 mM Tris-hydrochloric acid (pH 7.5) containing 1 mM EDTA). The flow-through fraction was applied to the column again for the same operation, and combined with the sample from the first operation. Using these procedures, 400 μg of poly(A+) RNA was obtained. The mRNA thus obtained was subjected to size fractionation by sucrose density gradient centrifugation as described in Schicif and Wensink's book on experimental techniques [Practical Methods in Molecular Biology, Springer-Verlag, New York, Heidelberg, Berlin (1981)].

Thus, in the tube for SW40Ti rotor (Beckman), a 5%-25% sucrose density gradient was prepared. The sucrose solutions were at 5% and 25% of sucrose (RNase-free, from Schwarz/Mann) in 10 mM Tris-hydrochloric acid (pH 7.5) containing 0.1 M NaCl, 1 mM EDTA and 0.5% SDS.

800 μg of the mRNA (poly(A+) RNA) prepared above was dissolved in 200 μL – 500 μL of TE buffer. After heating at 65°C for 5 min, the solution was cooled quickly and then layered on the sucrose density gradient. After centrifugation at 30,000 rpm for 20 hr, fractions of 0.5 mL each were collected and the absorbance at 260 nm was measured. From standard RNAs (28S, 18S and 5S ribosome RNAs) subjected to the same procedures, the sizes of the fractionated RNA were determined. Moreover, the G-CSF activity of each fraction was investigated using the Xenopus laevis oocyte system. Thus, mRNA from each fraction was prepared at 1 μg/μL in water, and then injected into oocytes isolated from Xenopus laevis (about one year old) at 50 ng mRNA/cell. The oocytes were placed in 96-well microtiter plate at 10 cells/well. 100 μL of culture medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 7.5 mM Tris-hydrochloric acid (pH 7.6), 10 mg/L of penicillin and 10 mg/L of streptomycin) were added to each well. After culture at room temperature for 48 hr, the supernatant was recovered. After concentration and purification, the G-CSF activity was assayed.

The results revealed G-CSF activity in the 15-17S fractions.

Practical Example 6: Synthesis of cDNA (construction of pBR-based cDNA library)

From the poly(A+) RNA prepared above, cDNA was obtained by Land et al.'s method [Nucleic Acids Res. Vol. 9, p. 2251 (1981)] with modifications from Gubier and Hoffman's method [Gene, Vol. 25, p. 263 (1983)].

1) Synthesis of single-stranded cDNA

In an Eppendorf's 1.5 mL tube, reagents were added in the following order: 80 μL of reaction buffer (500 mM KCl, 50 mM $MgCl_2$, 250 mM Tris-hydrochloric acid, pH 8.3), 20 μL of 200 mM dithiothreitol, 32 μL of 12.5 mM dNTP (dATP, dGTP, dCTP and dTTP each at 12.5 mM), 10 μL of α-32P-dCTP (Amersham, PB 10205), 32 μL of oligo(dT)12-18 (P-L Biochemicals, 500 μg/mL), 20 μL of poly(A+)-RNA (2.1 μg/μL), and 206 μL of distilled water (a total of 400 μL of the reaction mixture). After heating at 65°C for 5 min, the mixture was maintained at 42°C for 5 min. To this reaction mixture, 120 unit of a reverse transcriptase (Takara Syuzo) was added, followed by further reaction at 42°C for 2 hr. 2 μL of RNase inhibitor (Bethesda Research Laboratories), 20 μL of TE buffer, 16 μL of 100 mM sodium pyrophosphorate and 48 unit (4 μL) of the reverse transcriptase were further added, and then further reaction was performed at 46°C for 2 hr. 8 μL of 0.5 M EDTA and 8 μL of 10% SDS were added to stop the reaction. After phenol-chloroform treatment and ethanol precipitation (twice), a single-stranded cDNA was obtained.

2) dC addition to single-stranded cDNA

The single-stranded cDNA obtained above was dissolved in 60 μL of distilled water, and then 60 μL of a dC chain addition buffer (400 mM potassium cacodylate, 50 mM Tris-hydrochloric acid (pH 6.9), 4 mM dithiothreitol, 1 mM $CoCl_2$, and 1 mM dCTP) was added. After heating at 37°C for 5 min, 3 μL of a terminal transferase (27 unit/μL, P-L Biochemicals) was added, followed by reaction at 37°C for 2.5 min. After phenol-chloroform treatment (once) and ethanol precipitation (twice), the pellet was dissolved in 40 μL of TE buffer containing 100 mM NaCl.

3) Synthesis of double-stranded cDNA

To the above 40 μL of DNA solution, 4 μL of oligo(G)12-18 (200 μg/mL, P-L Biochemicals) was added, followed by heating at 65°C for 5 min and then at 42°C for 30 min. The reaction mixture was maintained at 0°C. To this reaction mixture, 80 μL of

buffer (100 mM Tris-hydrochloric acid, pH 7.5, 20 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 500 mM KCl), 4 μL of 4 mM dNTP (dATP, dGTP, dCTP and dTTP each at 4 mM), 60 μL of 1MMß-NAD, 210 μL of distilled water, 20 μL of E. coli DNA polymerase I (Takara Syuzo), 15 μL of E. coli DNA ligase (Takara Syuzo), and 15 μL of E. coli RNase H (Takara Syuzo) were added. After reaction at 12°C for 1 hr, 4 μL of 4 mM dNTP was further added, followed by further reaction at 25°C for 1 hr. After phenol-chloroform treatment and ethanol precipitation (once), about 8 μg of double-stranded cDNA was obtained. This double-stranded cDNA was dissolved in TE buffer and subjected to 1.2% agarose gel electrophoresis. Fractions corresponding to about 560 base pairs (bp) to 2 k base pairs (kbp) were eluted by adsorption on a Whatman DE81 filter paper (Whatman), thereby obtaining about 0.2 μg.

4) dC addition to double-stranded cDNA

The double-stranded cDNA obtained above was dissolved in 40 μL of TE buffer, and then 8 μL of the dC chain addition buffer described under 2) was added. After heating at 37°C for 2 min, 1 μL of a terminal transferase (27 unit/μL) was added, followed by reaction at 37°C for 3 min. The reaction solution was quickly cooled at 0°C and then 1 μL of 0.5 M EDTA was added to stop the reaction. After phenol-chloroform treatment and ethanol precipitation, the resultant pellet was dissolved in 10 μL of TE buffer.

5) Construction of pBR-based cDNA library

4 μL of a commercially available oligo(dG) chain-added pBR322 vector (Bethesda Research Laboratories, 10 ng/μL) was annealed with 2 μL of the above dC chain-added double-stranded cDNA in 75 μL of 0.1 M NaCl-containing TE buffer. Annealing was performed at 65°C for 5 min and then at 40°C for 2 hr, followed by standing until reaching room temperature.

On the other hand, the method described in Maniatis's book on experimental techniques [Molecular Cloning, Cold Spring Harbor, p. 249 (1982)] was used to prepare competent cells from E. coli strain X1776, to which the above annealed plasmid was transformed, thereby obtaining a transformant.

Practical Example 7: Synthesis of cDNA (construction of λ phage-based cDNA library)

1) Synthesis of single-stranded cDNA

By the method described in Practical Example 5, from 3.8 g of freeze-stored CHU-2 cells, 400 μg of poly(A+)-RNA was obtained after two rounds of purification on oligo(dT)-cellulose column.

10 μL of TE buffer with 12 μg of this poly(A+)-RNA dissolved was placed in a reaction tube containing 10 μg of actinomycin D (Sigma), and then reagents were added in the following order: 20 μL of reverse transcription buffer (250 mM Tris-hydrochloric acid, pH 8.3), 40 mM $MgCl_2$, 250 mM KCl), 20 μL 5 mM dNTP (dATP, dGTP, dCTP and dTTP each at 5 mM), 20 μL of oligo(dT)12-18 (0.2 μg/mL, P-L Biochemicals), 1 μL of 1 M dithiothreitol, 2 μL of 30 unit/μL RNase (Promega Biotech), 10 μL of reverse transcriptase (10 unit/μL, Seikagaku Kogyo), 1 μL of α-32P-dATP (10 μCi, Amersham), and 16 μL of water (a total of 100 μL of the reaction mixture). After heating at 42°C for 2 hr, 5 μL of 0.5 M EDTA and 1 μL of 20% SDS were added to stop the reaction. After phenol-chloroform treatment (100 μL) and ethanol precipitation (twice), about 4 μg of a single-stranded cDNA was obtained.

2) Synthesis of double-stranded cDNA

The cDNA obtained above was dissolved in 29 μL of TE buffer, and then reagents were added in the following order: 25 μL of polymerase buffer (400 mM Hepes (pH 7.6), 16 mM $MgCl_2$, 63 mM β-mercaptoethanol, 270 mM KCl), 10 μL of 5 mM dNTP, 1.0 μL of 15 mM β-NAD, 1.0 μL of α-32P-dATP (10 μCi/μL), 0.2 μL of E. coli DNA ligase (60 unit/μL, Takara Syuzo), 5.0 μL of E. coli DNA polymerase I (New England Biolabs, 10 unit/μL), 0.1 μL of E. coli RNase H (60 unit/μL, Takara Syuzo) and 28.7 μL of water.

After incubation at 14°C for 1 hr, the temperature was returned to room temperature, and the mixture was further incubated for 1 hr. Subsequently, 5 μL of 0.5 M EDTA and 1 μL of 20% SDS were added to stop the reaction. After phenol-chloroform treatment and ethanol precipitation, the resultant DNA was dissolved in 20 μL of 0.5 mM EDTA. 3 μL of Klenow buffer (500 mM Tris-hydrochloric acid (pH 8.0), 50 mM $MgCl_2$), 3 μL of 5 mM dNTP and 4 μL of water were added. To this reaction mixture, 1 μL of DNA polymerase (Klenow fragment) (Takara Syuzo) was added, followed by incubation at 30°C for 15 min.

The reaction mixture was diluted with 70 μL of TE buffer. Furthermore, 5 μL of 0.5 M EDTA and 1 μL of 20% SDS were added to stop the reaction. After phenol-chloroform treatment and ethanol precipitation, about 8 μg of double-stranded cDNA was obtained.

3) Methylation of double-stranded cDNA

30 μL of aqueous solution of the double-stranded cDNA synthesized in 2), 40 μL of methylation buffer (500 mM Tris-hydrochloric acid (pH 8.0), 50 mM EDTA), 20 μL of SAM solution (800 μM S-adenosyl-L-methylmethionine (SAM), 50 mM β-mercaptoethanol) and 100 μL of water were mixed. 15 μL of EcoRI methylase (New England Biolabs, 20 unit/μL) was added, making a total volume of 200 μL, followed by incubation at 37°C for 2 hr. After phenol treatment and ether treatment, ethanol precipitation was performed to recover the DNA.

4) Addition of EcoRI linker

To about 1.2 μg of the double-stranded DNA methylated above, 1.5 μL of ligase buffer (250 mM Tris-hydrochloric acid (pH 7.5), 100 mM $MgCl_2$), 0.5 μL of phosphate-oxidized(?) EcoRI linker (10 mer, Takara Syuzo), 1.5 μL of 10 mM ATP, 1.5 μL of 100 mM dithiothreitol, and 2 μL of water were added. To the reaction mixture in a volume of 15 μL, 0.7 μL of T4 DNA ligase (3.4 unit/μL, Takara Syuzo) was added, followed by reaction at 4°C overnight. The ligase was inactivated by heating at 65°C for 10 min. This reaction mixture was adjusted to a total volume of 50 μL with 100 mM Tris-hydrochloric acid (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 100 μg/mL gelatin, and then 3.5 μL of EcoRI (10 unit/μL) was added. After reaction at 37°C for 2 hr, 2.5 μL of 0.5 M EDTA and 0.5 μL of 20% SDS were added. After phenol-chloroform treatment, ethanol precipitation was performed to recover the DNA. Subsequently, by gel filtration on Ultrogel ACA34 (LKB) or agarose gel electrophoresis unreacted EcoRI linker was removed, thereby obtaining 0.5-0.7 μg of a linker-added double-stranded cDNA.

5) Ligation between double-stranded cDNA and λgt10 vector

The above linker-added double-stranded cDNA was mixed with 2.4 μg of EcoRI-treated λgt10 vector (Vector Cloning System), 1.4 μL of ligase buffer (250 mM Tris-hydrochloric acid, 100 mM $MgCl_2$), and 6.5 μL of distilled water. After treatment at 42°C for 15 min, 1 μL of 10 mM ATP, 1 μL of 0.1 M dithiothreitol, and 0.5 μL of T4

DNA ligase were added, making a total volume of 15 μL, followed by overnight reaction at 12°C.

6) In vitro packaging

About 1/3 of the recombinant DNA obtained in above 5) was packaged using an in-vitro packaging kit (Promega Biotech), thereby obtaining a phage plaque.

Practical Example 8: Screening of pBR-based library with probe (IWQ)

A Whatman No. 541 filter paper was placed on an agar medium with colonies grown. After 2 hr at 37°C, the filter paper was treated by Taub and Thompson's method [Anal. Biochem. Vol. 126, p. 222 (1982).

Thus, after the colonies were transferred to the No. 541 filter paper, they were incubated in an agar medium containing chloramphenicol (250 μg/μL) at 37°C overnight. The No. 541 filter paper was removed, and placed on a filter paper that had been soaked in 0.5 M NaOH solution at room temperature for 3 min. The process was repeated twice. Then, the same procedures were performed twice with 0.5 M Tris-hydrochloric acid (pH 8) solution for 3 min. Furthermore, at 4°C treatment was performed with 0.05 M Tris-hydrochloric acid (pH 8) solution for 3 min, with 1.5 mg/mL lysozyme solution (0.05 M Tris-hydrochloric acid (pH 8), containing 25% sucrose) for 10 min, and then at 37°C with 1 x SSC (0.15 M NaCl and 0.015 M sodium citrate) solution for 2 min, 1 x SSC solution containing 200 μg/mL protease K for 30 min, followed by again at room temperature with 1 x SSC for 2 min and with 95% ethanol solution for 2 min twice.

The No. 541 filter paper was dried. The resultant No. 541 filter paper was soaked in phenol : chloroform : isoamyl alcohol (25 : 24 : 1, equilibrated with 100 mM Tris-hydrochloric acid (pH 8.5), 100 mM NaCl, 10 mM EDTA) solution for 30 min. Then, the same procedures were performed with 5 x SSC for 3 min three times, and then with 95% ethanol solution for 3 min twice, followed by drying of the filter paper.

Probe (IWQ) was radio labeled with ^{32}P by the standard method (see Molecular Cloning), and then colony hybridization was performed by Wallace et al.'s method [Nucleic Acids Res. Vol. 9, p. 879 (1981)]. After pre-hybridization in a hybridization solution containing 6 x NET [0.9 M NaCl, 0.09 M Tris-hydrochloric acid (pH 7.5), 6 mM EDTA], 5 x Denhardt solution, 0.1% SDS, 0.1 mg/mL denatured DNA (fetal bovine thymus DNA) at 65°C for 4 hr, the above hybridization buffer containing the

radiolabeled probe (IWQ) at 1 x 10^6 cpm/mL was used to perform hybridization at 56°C overnight. After reaction, the No. 541 filter paper was washed at room temperature with 6 x SSC solution containing 0.1% SDS for 30 min, twice and then at 56°C for 1.5 min, followed by autoradiography.

From clones with a signal, plasmids were isolated. Probe (IWQ) was used to perform Southern blotting. Hybridization and autoradiography were performed under the same conditions as above.

Similarly, probe (A) was used to perform Southern blotting. Hybridization was performed using the above hybridization buffer at 49°C for 1 hr, and then after cooling to 39°C, at 39°C for 1 hr. After reaction, the nitrocellulose filter paper was washed at room temperature twice with 6 x SSC solution containing 0.1% SDS for 30 min, and then at 39°C for 3 min, followed by autoradiography.

The results revealed that one clone was positive. The nucleotide sequence was determined by the dideoxy method to be as shown in Fig. 2. It was a DNA comprising 308 base pairs, containing both probe (IWQ) and probe (A) sequences. The pBR322-derived plasmid containing this insert was named as PHCS-1.

Practical Example 9: Screening of λ phage-based cDNA library using PHCS-1-derived DNA probe

Plaque hybridization was performed in accordance with Benton and Davis's method [Science, Vol 196, p. 180 (1977)]. PHCS-1 obtained in Practical Example 8 was treated with Sau3A and EcoRI to obtain an about 600 base pair DNA fragment. This DNA fragment was radiolabeled by nick translation by the standard method. A nitrocellulose filter paper (S & S) was placed on an agar medium with phage plaques grown to transfer the phages. After denaturing DNA with 0.5 M NaOH, the filter paper was treated in the following order: 0.1 M NaCl and 1.5 M NaCl for 20 sec, 0.5 M Tris-hydrochloric acid (pH 7.5), 1.5 M NaCl for 20 sec twice, and finally 120 mM NaCl, 15 mM sodium citrate, 13 mM KH_2PO_4 and 1 mM EDTA (pH 7.2) for 20 sec.

Subsequently, the filter paper was dried, and heated at 80°C for 2 hr to immobilize DNA. Pre-hybridization was performed in a hybridization solution containing 5 x SSC, 5 x Denhardt solution, 50 mM phosphate buffer, 50% formamide, 0.25 mg/mL denatured DNA (salmon sperm DNA) and 0.1% SDS at 42°C overnight.

Hybridization was performed in hybridization buffer (5 x SSC, 5 x Denhardt solution, 20 mM phosphate buffer (pH 6.0), 50% formamide, 0.1% SDS, 10% dextran sulfate and 0.1 mg/mL denatured DNA (salmon sperm DNA) at 42°C for 20 hr.

The nitrocellulose filter paper was washed at room temperature with 2 x SSC solution containing 0.1% SDS for 20 min, and then at 44°C with 0.1 x SSC containing 0.1% SDS for 30 min and furthermore at room temperature with 0.1 x SSC for 10 min, followed by detection by autoradiography.

The results revealed that five clones were positive (G1-5). DNA sequence of a clone considered to contain a full-length cDNA was determined, thereby obtaining the nucleotide sequence shown in Fig. 3. This cDNA was cut out of the λgt10 vector, and ligated with pBR327 [Soberon et al.: Gene, Vol. 9, p. 287 (1980)] at the EcoRI site. The plasmid was prepared in a large quantity, and this plasmid was named pBRG4.

Practical Example 10: Screening λ phage-based cDNA library using pBRG4-derived DNA probe and probe (LC)

Plaque hybridization was performed in accordance with the Benton and Davis's method used in Practical Example 9 (see above mentioned reference). A nitrocellulose filter paper (S & S) was placed on an agar medium with phage plaques grown to transfer the phages. After denaturing DNA with 0.5 M NaCl, the filter paper was treated in the following order: 0.1 M NaOH and 1.5 M NaCl for 20 sec, 0.5 M Tris-hydrochloric acid (pH 7.5), 1.5 M NaCl for 20 sec twice, and finally 120 mM NaCl, 15 mM sodium citrate, 13 mM KH_2PO_4 and 1 mM EDTA (pH 7.2) for 20 sec. Subsequently, the filter paper was dried, and heated at 80°C for 2 hr to immobilize DNA. Two identical filter papers were prepared in this way, for screening with pBRG4-derived DNA probe and probe (LC), respectively.

When the pBRG4-derived DNA probe was used, pBRG4 was treated with EcoRI to obtain a DNA fragment with about 1500 base pairs. This DNA fragment was radiolabeled by nick translation by a standard method. The above filter paper was subjected to pre-hybridization in a hybridization solution containing 5 x SSC, 5 x Denhardt solution, 50 mM phosphate buffer, 50% formamide, 0.25 mg/mL denatured DNA (salmon sperm DNA) and 0.1% SDS at 42°C overnight. Hybridization was performed in hybridization buffer [(5 x SSC, 5 x Denhardt solution, 20 mM phosphate buffer (pH 6.0), 50% formamide, 0.1% SDS, 10% dextran sulfate and 0.1 mg/mL

denatured DNA (salmon sperm DNA] containing the above radiolabeled about 1500 base pairs DNA probe (about 1 x 10^6 cpm/mL) at 42°C for 20 hr. The nitrocellulose filter paper was washed at room temperature with 2 x SSC solution containing 0.1% SDS for 20 min, and then at 44°C with 0.1 x SSC containing 0.1% SDS for 30 min and furthermore at room temperature with 0.1 x SSC for 10 min, followed by detection by autoradiography.

When a probe (LC) was used, the filter paper was subjected to pre-treatment in 3 x SSC containing 0.1% SDS at 65°C for 2 hr, and then to pre-hybridization in a buffer containing 6 x NET, 1 x Denhardt solution, 100 μg/mL denatured DNA (salmon sperm DNA) at 65°C for 2 hr.

Hybridization was performed in hybridization buffer [6 x NET, 1 x Denhardt solution, 100 μg/mL denatured DNA (salmon sperm DNA] containing the above radiolabeled probe (LC) (2 x 10^6 cpm/mL) at 63°C overnight. The nitrocellulose filter paper was washed at room temperature with 6 x SSC solution containing 0.1% SDS for 20 min. After washing 3 times with this solution, 6 x SSC containing 0.1% SDS was used for washing at 63°C for 2 min.

The filter was dried and then subjected to autoradiography for detection.

By the screening performed above, clones positive with both probes were selected. The cDNA sequence of a clone considered to contain a full-length cDNA was determined by the dideoxy method, thereby obtaining the nucleotide sequence shown in Fig. 4A. This cDNA was cut out of the λgt10 vector, and ligated with pBR327 at the EcoRI site, thereby obtaining a plasmid named pBRV2.

Practical Example 11: Example of use of tac promoter-containing vector

1) Construction of recombinant vector

(1) Preparation of vector

5 μg of the tac promoter-containing vector pKK223-3 (Pharmacia) was mixed with 30 μL of a reaction solution (50 mM Tris-HCl, 7 mM $MgCl_2$, 100 mM NaCl, 7 mM 2-mercaptoethanol) and the mixture was treated with 8 units of EcoRI (Takara Syuzo) at 37°C for 2 hr.

Subsequently, 3 μL of alkaline phosphatase (Takara Syuzo) was added and the mixture was treated at 60°C for 30 min. Phenol treatment was performed three times by the standard method. Ether treatment and ethanol precipitation were performed to recover the DNA fragment.

This DNA was dissolved in 50 μL of 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM DTT, 1 mM dATP, dCTP, dGTP and TTP, and 3 μL of E. coli DNA polymerase I-Klenow fragment (Takara Syuzo) was added. By reaction at 14°C for 2 hr, the terminal was converted to a blunt end.

(2) Preparation of synthetic linker

3 μg of a synthetic linker, an oligonucleotide with the sequence CGAATGACCCCCCT GGGCC and CAGGGGGGTCATTCG, was treated in 40 μL of 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 1 mM ATP with 4 units of T_4 polynucleotide kinase at 37°C for 60 min for phosphorylation. Then, 0.2 μg of the phosphorylated oligonucleotide was dissolved in 20 μL of TE (10 mM Tris-HCl, pH 8, 0.1 mM EDTA) containing 100 mM NaCl, and treated at 65°C for 10 min, followed by gradual cooling to room temperature for annealing.

(3) Preparation of cDNA fragment of G-CSF

60 μg of the pBRV2 containing the cDNA shown in Fig. 4(A) was treated with 100 units of the restriction enzyme ApaI (New England Biolabs) and 50 units of DraI (Takara Syuzo) in 200 μL of 6 mM Tris-HCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol at 37°C for 3 hr. By 1.2% agarose gel electrophoresis, about 2 μg of a ApaI-DraI fragment with about 590 bp was recovered.

(4) Ligation of the above fragments

0.1 μg each of the fragments from (1), (2) and (3) were dissolved in 20 μL of a ligation solution (66 mM Tris-HCl, 6.6 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and then 175 units of T4 DNA ligase were added. After reaction overnight at 4°C, a recombinant vector was obtained.

2) Transformation

20 μL of the reaction solution containing recombinant vector obtained in above (4) was transformed into E. coli strain JM105 by the rubidium chloride method [see

above mentioned reference: T. Maniatis et al.: Molecular Cloning, p. 252 (1982)]. The plasmid was isolated from the resultant transformant strain by culturing in an ampicillin-resistant colony culture medium. By treatment with the restriction enzymes BamHI, ACCII and ApaI, it was confirmed to be the target transformant.

Practical Example 12: [Example of use of PL promoter-containing vector]

1) Construction of recombinant vector

100 μg of the PL promoter-containing vector pPL-lambda (Pharmacia) was treated with 50 units of the restriction enzyme BamHI in 100 μL of a reaction solution (10 mM Tris-HCl, pH 7.6, 7 mM $MgCl_2$, 100 mM NaCl, 10 mM DTT) at 37°C overnight.

Then, by 1% agarose gel electrophoresis about 49 μg of an about 4 Kbp fragment and about 11 μg of an about 1.2 Kbp fragment were recovered.

Between the above fragments, first the about 4 Kbp one was dissolved in 100 μL of the above described TE buffer, and then allowed to react with 5 μL of alkaline phosphatase (Takara Syuzo) at 60°C for 60 min for dephosphorylation.

The other fragment, the about 1.2 Kbp one, was dissolved in 20 μL of a buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 6 mM KCl, 1 mM DTT) and then treated with 20 unit of the restriction enzyme MboII (New England Biolabs) at 37°C overnight.

Subsequently, using 4% polyacrylamide gel electrophoresis, about 0.9 μg of a BamHI-MboII fragment of about 200 bp and about 1.9 μg of a MboII-BamHI fragment of about 3100 bp were recovered.

(2) Preparation of synthetic linker

A synthetic linker, $-TAAGGAGAATTCATC\ GAT$ and $TCGATGAATTCTCCTT\ \ AG$, was phosphorylated and then annealed as in Practical Example 11 (2), thereby obtaining a synthetic linker S/D.

(3) Preparation of expression vector

0.1 μg of the above about 4 Kbp fragment prepared in (1), the BamHI-MboII

fragment with an O_LP_L region and the MboII-BamHI fragment with a tL_1 region, each 0.05 μg, and 0.1 μg of the synthetic linker S/D were treated with 175 units of T4 DNA ligase (Takara Syuzo) in 40 μL of a reaction solution (66 mM Tris-HCl, 6.6 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) at 12°C overnight.

20 μL of this reaction solution was used for transformation in E. coli strain N99cI (Pharmacia) by the $CaCl_2$ method (see above mentioned "Molecular Cloning").

The transformant was cultured, and the plasmid was isolated from ampicillin-resistant colony culture. Then the plasmid was confirmed by treatment with the restriction enzymes EcoRI, BamHI and SmaI.

Subsequently, 2 μg of this plasmid was treated with 5 units of the restriction enzyme ClaI (New England Biolabs) in 20 μL of a buffer (10 mM Tris-HCl, 6 mM HCl, 6 mM $MgCl_2$, 50 mM NaCl) at 37°C for 2 hr, followed by heating at 65°C for 10 min for inactivation.

Furthermore, 1 μL of the reaction solution was mixed with 20 μL of the above reaction solution and treated with 175 unit of T_4 DNA ligase (Takara Syuzo) at 12°C overnight. After transformation in E. coli strain N99cI (Pharmacia) again as above, the plasmid was isolated from ampicillin-resistant colony culture. The plasmid was confirmed by treatment with the restriction enzymes EcoRI and BamHI.

(4) Preparation of recombinant vector for G-CSF expression and of the transformant

The expression plasmid obtained in (3) was treated with the restriction enzyme ClaI and then the terminus was converted to a blunt end. A recombinant vector containing the G-CSF cDNA fragment was obtained as in Practical Example 11. This vector was used to transform E. coli strain N4830 (Pharmacia) by $CaCl_2$ method as described in the above-mentioned Molecular Cloning. Confirmation of the target transformant was also performed as in Practical Example 11.

Practical Example 13: Example of use of trp promoter-containing vector

1) Construction of recombinant vector

(1) Preparation of vector

10 μg of pOY1 plasmid prepared by inserting a HpaII-TaqI fragment of about 330 bp containing a tryptophan promoter at the ClaI site of pBR322 was mixed with 30 μL of

a reaction solution (10 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl) and the mixture was treated with 7 units of the restriction enzyme ClaI and 8 unit of PvuII at 37°C for 3 hr. Subsequently, 2 μL of alkaline phosphatase (Takara Syuzo) was added and the mixture was treated at 60°C for 1 hr.

By 1% agarose gel electrophoresis about 2.5 μg of an about 2.6 Kbp fragment was recovered.

(2) Preparation of synthetic linker

The synthetic linker

−CGCGAATGACCCCCCTGGGCC and

CAGGGGGGGTCATTCG was phosphorylated and annealed as in (2) of Practical Example 11.

(3) Preparation of recombinant vector

About 1 μg of the above vector prepared in (1) and about 1 μg of the synthetic linker of (2), and about 1 μg of the G-CSF cDNA fragment prepared in (3) of Practical Example 11 were treated with 175 units of T4 DNA ligase (Takara Syuzo) in 20 μL of the above ligation solution at 12°C over-night, thereby obtaining a recombinant vector.

2) Transformation

20 μL of the above reaction solution of above (3) was transformed into E. coli strain DH1 by calcium chloride method as described in the above mentioned Molecular Cloning.

As in Practical Example 11, the plasmid was obtained from ampicillin-resistant colony and the target transformant was confirmed by treatment with the restriction enzymes ApaI, DraI, NruI and PstI.

Practical Example 14: Culture of transformant strain

1) Culture of the transformant strain (containing Tac) obtained in Practical Example 11

1 mL of the transformant strain cultured over-night at 37°C was added to 100 mL of Luria culture medium containing 25 μg/mL or 50 μg/mL of ampicillin, and then cultured at 37°C for 2-3 hr. Then, isopropyl-β-D-thiogalactoside was adjusted to 2 mM, followed by culture at 37°C for 2-4 hr.